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# Osmotic stress tolerance in semi-terrestrial tardigrades

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Little is known about ionic and osmotic stress tolerance in tardigrades. Here, we examine salt stress tolerance in *Ramazzottius oberhaeuseri* and *Echiniscus testudo* from Nivå (Denmark) and address whether limno-terrestrial tardigrades can enter a state of quiescence (osmobiosis) in the face of high external osmolyte concentrations. Direct transfers into NaCl solutions showed an upper tolerance level of around 600 mOsm kg<sup>-1</sup> in *R. oberhaeuseri* and 200 mOsm kg<sup>-1</sup> in *E. testudo*. During salt exposures, *R. oberhaeuseri* contracted into a ‘tun’, whereas *E. testudo* remained active leaving it more susceptible to acute effects of the ions. Further experiments focused on the more resilient *R. oberhaeuseri*, which entered a tun and readily regained activity when directly exposed to polyethylene glycol and sucrose of up to 872 ± 0 and 813 ± 3 mOsm kg<sup>-1</sup>, respectively, revealing a higher tolerance towards non-ionic osmolytes as compared to NaCl. *Ramazzottius oberhaeuseri* furthermore readily regained activity following gradual increases in non-ionic osmolytes and NaCl of up to 2434 ± 28 and 1905 ± 3 mOsm kg<sup>-1</sup>, respectively, showing that short-term acclimation promoted salt stress tolerance. Our results suggest that the limno-terrestrial *R. oberhaeuseri* enters a state of quiescence in the face of high external osmotic pressure and that it, in this state, is highly tolerant of ionic and osmotic stress.

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**ADDITIONAL KEYWORDS:** cryptobiosis – Ecdysozoa – extreme environments – ionic stress – osmobiosis.

## INTRODUCTION

Tardigrades are renowned for their abilities to survive in extreme environments and many recent investigations address the limits of their tolerance, for example in relation to dehydration, freezing, heating and radiation (e.g. Jönsson & Bertolani, 2001; Horikawa *et al.*, 2006; Jönsson *et al.*, 2008; Møbjerg *et al.*, 2011; Persson *et al.*, 2011; Węlnicz *et al.*, 2011). These microscopic metazoans survive extremes by entering

dormant states, such as cryptobiosis, a state of latent life characterized by a reversible shutdown of metabolism (Sømme, 1996; Clegg, 2001; Wharton, 2015). Keilin (1959) originally defined four versions of cryptobiosis: anhydrobiosis (induced by dehydration), cryobiosis (induced by cooling), anoxybiosis (induced by lack of oxygen) and osmobiosis (induced by high salt concentration). Salts dissociate into ions that build osmotic pressures and osmobiosis would thus allow tardigrades to enter a state of quiescence (metabolic shut-down) in the face of extreme perturbations in external ionic strength and osmotic pressure. However, little is known of osmobiosis and it has been

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argued that this form of cryptobiosis may not exist in tardigrades (Guidetti, Altiero & Rebecchi, 2011).

The phylum Tardigrada is divided into two main evolutionary lineages, separated by distinct molecular and morphological traits: Heterotardigrada and Eutardigrada (Sands *et al.*, 2008; Jørgensen *et al.*, 2010). Members of both lineages thrive in marine, limnic and terrestrial environments, yet all species need a film of water to be in their active state. A significant difference between marine and limnic/semi-terrestrial habitats is the amount of salts present in the surrounding water. Thus, ionic and osmotic stress tolerance may represent a key driver in the evolution of tardigrades (Møbjerg, Kristensen & Jørgensen, 2016). Regardless of this possible significance, only a limited number of studies have been performed on salt and osmotic stress tolerance in tardigrades, mainly including marine species (Halberg *et al.*, 2009; Jørgensen & Møbjerg, 2015; Hygum *et al.*, 2016). During a rise in external ionic strength and osmotic pressure, some marine heterotardigrades contract into a so-called 'tun', characterized by a longitudinal contraction of the body and a withdrawal of limbs (Jørgensen & Møbjerg, 2015; Hygum *et al.*, 2016). The tun-state is best known from limno-terrestrial species that rely on this compact body shape for anhydrobiotic survival (e.g. Sømme, 1996; Bertolani *et al.*, 2004; Wełnicz *et al.*, 2011; Halberg, Jørgensen & Møbjerg, 2013a). However, tun formation has also been observed in limno-terrestrial eutardigrades following exposure to various salts (Collin & May, 1950). In the current study, we investigate salt stress tolerance in two limno-terrestrial species, namely the eutardigrade *Ramazzottius oberhaeuseri* (Doyère, 1840) and the heterotardigrade *Echiniscus testudo* (Doyère, 1840), and address whether limno-terrestrial tardigrades can enter a state of osmobiosis.

## MATERIAL AND METHODS

### TARDIGRADE COLLECTION AND HANDLING

Moss and sediment containing the tardigrades *Ramazzottius oberhaeuseri* and *Echiniscus testudo* were collected from a roof gutter and roof top in Nivå, Denmark (55°56'36.53"N, 12°30'00.90"E) during the period August 2014 to July 2015. The samples were kept refrigerated at approximately 5 °C in plastic or glass containers with tap water for up to 2 weeks. Tardigrades were sorted from this stock solution using a dissection microscope (Stemi 2000; Carl Zeiss International, Jena, Germany) and transferred into watch glasses with ultrapure water (Barnstead EASYpure UV/UF, Dubuque, IA, USA; and Millipore Milli-Q® Reference, Merck, Darmstadt, Germany), where they were kept for up to 1 week. Along with

the animals, small amounts of moss/sediment were deliberately added, in order for the tardigrades to have something to eat/hold on to and not spend too much energy searching for food/substrate (Schill *et al.*, 2011). Immediately prior to experimentation groups ( $N = 3-6$ ) of approximately 20 animals were cleared of substrate and transferred to a 12-well Nunc plate. Tardigrade activity was monitored under a microscope in purified water immediately prior to osmolyte exposure ( $t = 0$  h), after the 24-h exposure to test solutions at 5 °C ( $t = 24$  h), and three times after retransfer to purified water ( $t = 26$  h,  $t = 48$  h and  $t = 72$  h). For each experimental series, control groups ( $N = 3-6$ ) were kept in purified water in Nunc plates at 5 °C for the entire period. Tardigrades were considered active when they showed clear movement or were responsive to tactile stimuli.

### PREPARATION OF EXPERIMENTAL SOLUTIONS

Nine NaCl test solutions were prepared from a 1 mol l<sup>-1</sup> stock solution and the osmolalities (mOsm kg<sup>-1</sup>) of the solutions were subsequently measured using freezing point depression (Advanced Model 3320 Micro-Osmometer, Advanced Instruments):  $93 \pm 0$ ,  $192 \pm 1$ ,  $281 \pm 0$ ,  $463 \pm 1$ ,  $560 \pm 1$ ,  $758 \pm 1$ ,  $941 \pm 1$ ,  $1447 \pm 2$  and  $1905 \pm 3$  (mean  $\pm$  SEM;  $N = 3-6$ ).

For a second set of experiments on *R. oberhaeuseri*, nine sucrose and PEG-400 [poly(ethylene glycol), average Mn 400] solutions were prepared with calculated molalities of (mmol kg<sup>-1</sup>): 100, 200, 300, 500, 600, 800, 1000, 1500 and 2000. The osmotic activities of the 100 and 200 mmol kg<sup>-1</sup> solutions were measured directly by freezing point depression, whereas measurements for all other PEG-400 and sucrose solutions were made on dilutions of the given solution to a theoretical molality of 100 mmol kg<sup>-1</sup>. The osmolalities of the solutions were subsequently estimated based on the assumption of a linear relation between molal concentration and osmolality, which probably means that the osmotic activities of the PEG-400 solutions are underestimated (e.g. Schiller *et al.*, 1988; Money, 1989; Kiyosawa, 2003). The nine PEG-400 solutions thus had the following estimated osmotic activities (mOsm kg<sup>-1</sup>):  $108 \pm 3$ ,  $218 \pm 1$ ,  $348 \pm 0$ ,  $583 \pm 9$ ,  $694 \pm 2$ ,  $872 \pm 0$ ,  $1060 \pm 6$ ,  $1856 \pm 10$  and  $2434 \pm 28$  (mean  $\pm$  SEM;  $N = 3-7$ ), and the nine sucrose solutions (mOsm kg<sup>-1</sup>):  $94 \pm 1$ ,  $197 \pm 1$ ,  $307 \pm 1$ ,  $497 \pm 3$ ,  $604 \pm 7$ ,  $813 \pm 3$ ,  $1027 \pm 3$ ,  $1630 \pm 6$  and  $2060 \pm 12$  (mean  $\pm$  SEM;  $N = 3$ ).

### DATA ANALYSIS AND PRESENTATION OF RESULTS

Data on tardigrade activity are represented as means of activity percentages using means  $\pm$  standard errors

of the mean (SEM). The activity (as a percentage) of each group was calculated based on the number of active specimens divided by the total number of specimens in the group (~20 animals in each group). A few statistical comparisons have been used to evaluate interpretations, where these are not immediately obvious. For these analyses, the software OriginPro 9 (OriginLab) was used to test for normality (Shapiro–Wilk) and variance ( $F$ -test) before computing either a  $t$ -test or a Mann–Whitney  $U$ -test. Light micrographs were taken with a Leica DM1000 microscope equipped with an Infinity X digital camera (DeltaPix, Smørum, Denmark). Figures were made in OriginPro 9 and CorelDRAW X7 (Corel Corporation, Ottawa, Canada).

## RESULTS AND DISCUSSION

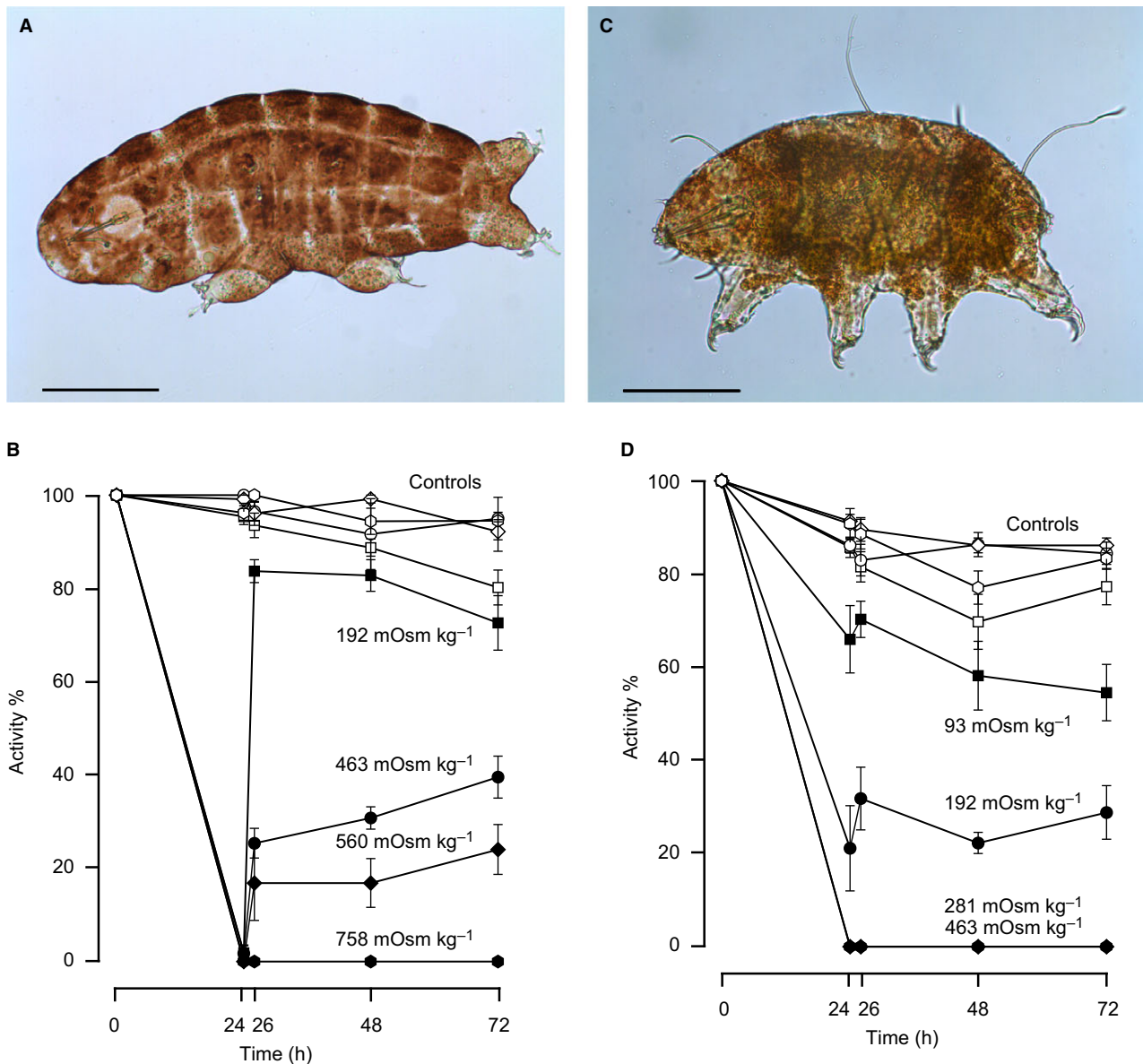
The tardigrades *Ramazzottius oberhaeuseri* and *Echiniscus testudo* originate from an extreme environment, i.e. moss and sediment from a roof in Denmark. This habitat is subjected to frequent dehydration as well as subzero temperatures during winter. Both species must therefore be able to withstand dehydration as well as freezing. In the current study we investigate salt and osmotic stress tolerance in these limno-terrestrial species.

Our initial experiments showed that *Ramazzottius oberhaeuseri* (Fig. 1A, B) ceased to be active and contracted into a tun following acute NaCl exposure. Thus, the activity of *R. oberhaeuseri* dropped from  $100 \pm 0\%$  to  $2 \pm 2\%$  following exposure to  $192 \pm 1$  mOsm  $\text{kg}^{-1}$ . However, 2 h after retransfer to purified water the activity ( $84 \pm 2\%$  at  $t = 26$  h) increased to a level comparable to the control animals and it remained at a high level until the end of experimentation. Exposure to  $463 \pm 1$  and  $560 \pm 1$  mOsm  $\text{kg}^{-1}$  resulted in a decrease in activity from  $100 \pm 0\%$  to  $2 \pm 2\%$  and  $0 \pm 0\%$ , respectively. Following retransfer to purified water, activity increased to  $40 \pm 5\%$  and  $24 \pm 5\%$  at  $t = 72$  h. Exposure to  $758 \pm 1$  mOsm  $\text{kg}^{-1}$  abolished activity, which was not regained following retransfer to purified water ( $0\%$  activity at  $t = 72$  h). Thus, the upper tolerance level of *R. oberhaeuseri*, when exposed directly to high concentration NaCl solutions, was around 600 mOsm  $\text{kg}^{-1}$ . This value is in the range of the upper lethal limit (around 500 mOsm  $\text{kg}^{-1}$ ) for another limno-terrestrial eutardigrade, i.e. *Richtersius coronifer* (Richters, 1903) (Bjørn-Mortensen, 2006; Møbjerg *et al.*, 2011). Interestingly, osmolality experiments with NaCl vs. PEG showed that *R. coronifer* is more vulnerable to short-term fluctuations with NaCl as osmolyte than with the non-ionic PEG (Bjørn-Mortensen, 2006). This is consistent with the notion that NaCl has effects beyond the pure osmolytic activity.

Our experiments showed that *Echiniscus testudo* was more sensitive to NaCl-induced stress (Fig. 1C, D). The activity (mean  $\pm$  SEM) of this heterotardigrade dropped from  $100 \pm 0\%$  (at  $t = 0$  h) to  $66 \pm 7\%$  and  $21 \pm 9\%$  following 24 h of exposure to  $93 \pm 0$  and  $192 \pm 1$  mOsm  $\text{kg}^{-1}$  NaCl, respectively. Activity levels did not increase considerably following retransfer to purified water. No activity was observed following exposure to osmolarities of  $281 \pm 0$  and  $463 \pm 1$  mOsm  $\text{kg}^{-1}$  and *E. testudo* did not regain activity following retransfer to purified water from these solutions. Thus, the upper NaCl tolerance level of *E. testudo* seemed to be around 200 mOsm  $\text{kg}^{-1}$ . Notably, *E. testudo* remained active during the salt exposures and did not contract into a tun, as evidenced by the activity levels at  $t = 24$  (Fig. 1D), i.e. this species did not enter a state of metabolic shut-down under the current experimental conditions. An overall decline in the activity of controls ( $N = 15$ ), from  $100 \pm 0\%$  at  $t = 0$  to  $81 \pm 2\%$  at  $t = 72$  ( $P = 6 \times 10^{-9}$ , Mann–Whitney  $U$ -test), would indicate that *E. testudo* was sensitive either to our handling and/or exposure to purified water deprived of substrate. Thus, no further experimentation was performed on this species. It should be noted, however, that *E. testudo* is well known for its ability to enter the tun state and that it may stay quiescent in this state for many years (Jørgensen, Møbjerg & Kristensen, 2007).

We subsequently tested the effect on *R. oberhaeuseri* of direct transfers into non-ionic solutions of PEG-400 ( $218 \pm 1$  and  $872 \pm 0$  mOsm  $\text{kg}^{-1}$ ) and sucrose ( $197 \pm 1$  and  $813 \pm 3$  mOsm  $\text{kg}^{-1}$ ), as well as gradual transfers into high-osmolality solutions of these osmolytes (Fig. 2A, B). The latter transfers were performed with 2 h of acclimation in eight solutions of increasing osmolality (i.e. for PEG-400:  $108 \pm 3$ ,  $218 \pm 1$ ,  $348 \pm 0$ ,  $583 \pm 9$ ,  $694 \pm 2$ ,  $872 \pm 0$ ,  $1060 \pm 6$ ,  $1856 \pm 10$  mOsm  $\text{kg}^{-1}$  and for sucrose:  $94 \pm 1$ ,  $197 \pm 1$ ,  $307 \pm 1$ ,  $497 \pm 3$ ,  $604 \pm 7$ ,  $813 \pm 3$ ,  $1027 \pm 3$ ,  $1630 \pm 6$  mOsm  $\text{kg}^{-1}$ ) followed by 8 h in  $2434 \pm 28$  mOsm  $\text{kg}^{-1}$  PEG-400 and  $2060 \pm 12$  mOsm  $\text{kg}^{-1}$  sucrose, respectively. *Ramazzottius oberhaeuseri* contracted into a tun and became inactive within a few minutes after being transferred into the high osmolyte solutions. After 24 h of exposure to these non-ionic osmolytes, activity was  $0\%$  in all cases except the  $197 \pm 1$  mOsm  $\text{kg}^{-1}$  sucrose solution ( $3 \pm 2\%$  activity at  $t = 24$  h). Following retransfer to purified water, activity ( $t = 26$  h) was at an overall average of  $97 \pm 1\%$ , and it stayed at this high level throughout experimentation. This implies that *R. oberhaeuseri* entered a state of quiescence during transfers into the non-ionic osmolytes and that it, upon return to purified water, regained full activity.

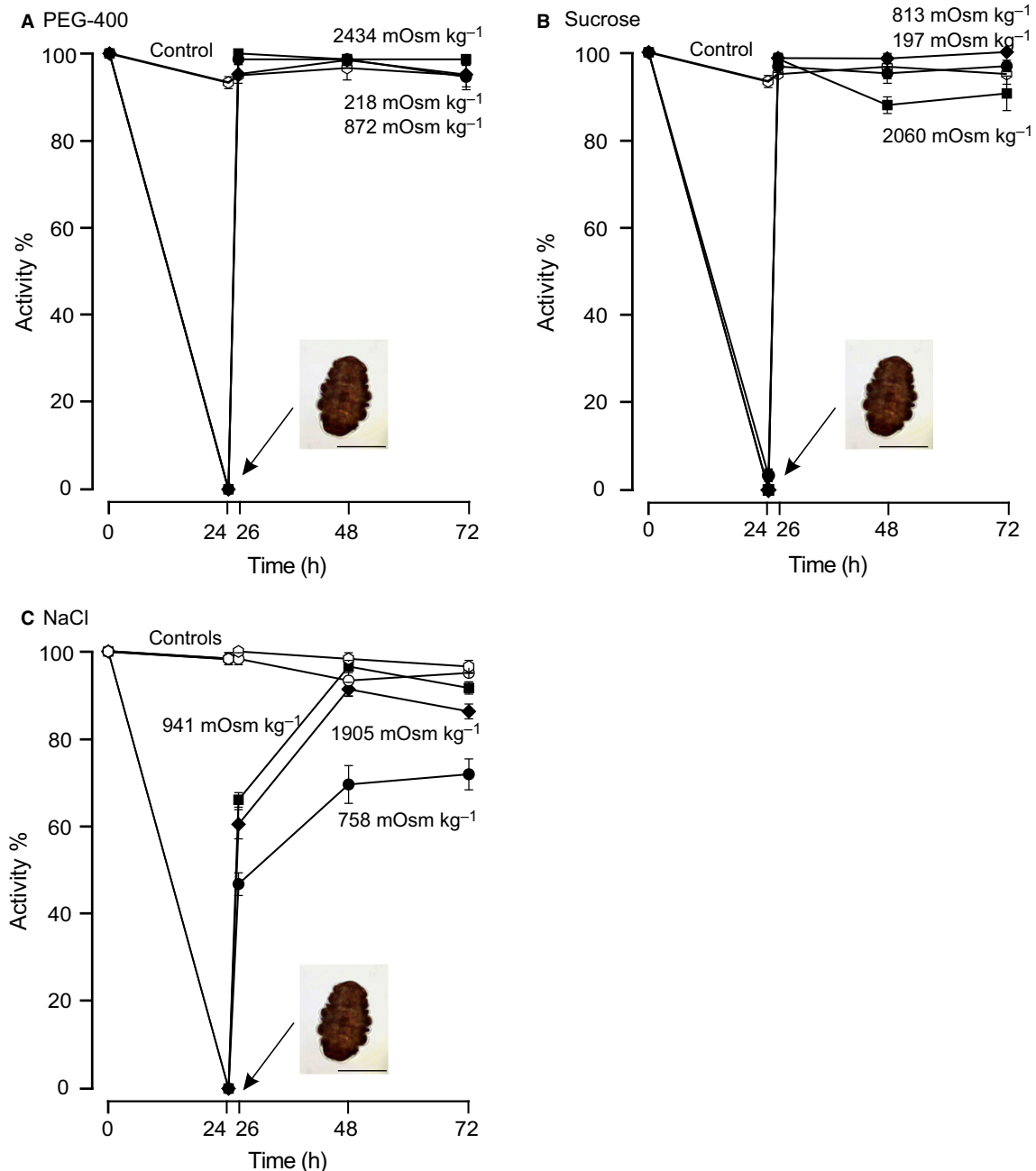




**Figure 1.** A, Light micrograph of *Ramazzottius oberhaeuseri*. Scale bar = 100  $\mu\text{m}$ . B, Activity in percentage (mean  $\pm$  SEM) of *R. oberhaeuseri* in experimental series ( $N = 3-6$ ) with acute exposures to  $192 \pm 1$ ,  $463 \pm 1$ ,  $560 \pm 1$  and  $758 \pm 1$  mOsm kg<sup>-1</sup> NaCl solutions. The top four graph lines (open symbols) express the activity in percentage (mean  $\pm$  SEM) of the corresponding control groups ( $N = 3-6$ ) in purified water. C, Light micrograph of *Echiniscus testudo*. Scale bar = 100  $\mu\text{m}$ . D, Activity in percentage (mean  $\pm$  SEM) of *Echiniscus testudo* in experimental series ( $N = 3-6$ ) with acute exposures to  $93 \pm 0$ ,  $192 \pm 1$ ,  $281 \pm 0$  and  $463 \pm 1$  mOsm kg<sup>-1</sup> NaCl solutions. The top four graph lines (open symbols) express the activity in percentage (mean  $\pm$  SEM) of the corresponding control groups ( $N = 3-6$ ) in purified water. In B and D  $t = 0$  h represents the activity in purified water immediately prior to osmolyte exposure, whereas  $t = 24$  h indicates the activity after exposure to a given test solution for 24 h. The tardigrades were subsequently transferred to purified water and their activity was assessed at  $t = 26$  h,  $t = 48$  h and  $t = 72$  h.

To investigate whether short-term acclimation to NaCl would promote tolerance in *R. oberhaeuseri*, experiments were performed in which tardigrades were exposed to gradual increases in external NaCl

(Fig. 2C). Specifically, *R. oberhaeuseri* was exposed to NaCl solutions starting at  $93 \pm 0$  mOsm kg<sup>-1</sup> followed by a change to higher osmolality every 2 h to final solutions of  $758 \pm 1$ ,  $941 \pm 1$  and



**Figure 2.** A, Activity in percentage (mean  $\pm$  SEM;  $N = 3$ ) of *Ramazzottius oberhaeuseri* exposed to PEG-400 solutions of  $218 \pm 1$  and  $872 \pm 0$  mOsm kg<sup>-1</sup>, as well as to gradual transfers into a solution of  $2434 \pm 28$  mOsm kg<sup>-1</sup>. Top graph line (open symbol) expresses the activity in percentage (mean  $\pm$  SEM) of the control groups ( $N = 3$ ) kept in purified water. B, Activity in percentage (mean  $\pm$  SEM;  $N = 3$ ) of *R. oberhaeuseri* exposed to sucrose solutions of  $197 \pm 1$  and  $813 \pm 3$  mOsm kg<sup>-1</sup>, as well as to gradual transfers into a solution of  $2060 \pm 12$  mOsm kg<sup>-1</sup>. Top graph line (open symbol) expresses the activity (mean  $\pm$  SEM) of the control groups ( $N = 3$ ) kept in purified water. C, Activity in percentage (mean  $\pm$  SEM;  $N = 3$ ) of *R. oberhaeuseri* exposed to a gradual increase in NaCl concentrations to a final osmolality of  $758 \pm 1$ ,  $941 \pm 1$  and  $1905 \pm 3$  mOsm kg<sup>-1</sup>, respectively. Top graph lines (open symbols) show the activity in percentage (mean  $\pm$  SEM) of two sets of control groups ( $N = 3$  in each set) kept in purified water. Inserted: *R. oberhaeuseri* contracted into a 'tun' during solute exposures. Scale bar = 100  $\mu$ m. All pictures show a tun induced by a  $463 \pm 1$  mOsm kg<sup>-1</sup> NaCl exposure. In all graphs  $t = 0$  h represents the activity in purified water immediately prior to osmolyte exposure, whereas  $t = 24$  h indicates the activity after exposure to the test solutions for 24 h. The animals were subsequently transferred to purified water and their activity was assessed at  $t = 26$  h,  $t = 48$  h and  $t = 72$  h.

$1905 \pm 3$  mOsm  $\text{kg}^{-1}$ , respectively. Our results showed that short-term acclimation promoted stress tolerance. Specifically, activity was  $72 \pm 4\%$  ( $N = 3$ ) at  $t = 72$  h following exposure to  $758 \pm 1$  mOsm  $\text{kg}^{-1}$  ( $P = 0.002$ ; one-tailed, one sample  $t$ -test, assuming a theoretical activity of 0% for non-acclimated animals). Activities following the gradual exposures to  $941 \pm 1$  and  $1905 \pm 3$  mOsm  $\text{kg}^{-1}$  were  $92 \pm 1\%$  and  $86 \pm 1\%$ , respectively, at  $t = 72$  h.

In comparison, a salinity tolerance test on *Caenorhabditis elegans* Maupas, 1900 by Khanna *et al.* (1997) revealed that this widely studied nematode tolerated up to 20 ‰ (20.5 g  $\text{l}^{-1}$  NaCl) for 24 h without any significant increase in mortality. This salinity would correspond to around 650 mOsm  $\text{kg}^{-1}$  (see e.g. Halberg *et al.*, 2009). It should be noted that the protocols used in the current study and the one used by Khanna *et al.* (1997) were not directly comparable. Nevertheless, it would seem that *C. elegans* is notably less tolerant than *R. oberhaeuseri*. Much lower salt tolerance levels are found among, for example, daphnids with reported  $\text{LC}_{50}$  values for NaCl, at the end of a three-brood test, of 4.2 ‰ for *Daphnia magna* Strauss, 1820 and 2.2 ‰ for *Ceriodaphnia dubia* Richard, 1894 (Cowgill & Milazzo, 1991).

Our results show that limno-terrestrial tardigrades are highly tolerant of ionic and osmotic stress. Both investigated tardigrades were sensitive to acute NaCl stress, confirming the assumption that exposure to  $\text{Na}^+$  and  $\text{Cl}^-$  ions has effects on tissues and organs apart from their pure osmolytic activity. The latter is confirmed by our results on *R. oberhaeuseri*, revealing an extreme tolerance towards high concentrations of non-ionic osmolytes, i.e. PEG and sucrose. Notably,  $\text{Na}^+$  and  $\text{Cl}^-$  ions are major constituents of animal body fluids (e.g. Halberg *et al.*, 2013b), and normal body function (e.g. muscle contraction and nerve impulses) relies on controlling the extracellular concentration of these ions. The latter is achieved through proteins embedded in the plasma membranes of specialized epithelial cells (e.g. Halberg & Møbjerg, 2012). Our experiments on *R. oberhaeuseri* revealed that acclimation to NaCl, i.e. a gradual exposure to increasing ion concentrations, promoted tolerance. Thus, this limno-terrestrial tardigrade can handle extreme external salt concentrations when given time to protect its tissues and organs from the associated perturbations in internal  $\text{Na}^+$  and  $\text{Cl}^-$  concentration. Importantly, during the exposures to hyperosmotic solutions the tardigrade contracted into the inactive tun state and in this state it was highly tolerant of osmotic as well as ionic stress (Fig. 2). The latter suggests that *R. oberhaeuseri* may enter osmobiosis as a response to elevated external osmotic pressures.

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